Forty years of assisted reproduction research in non-domestic, wild and endangered mammals

40 anos de pesquisa em reprodução de mamíferos não-domésticos, selvagens e em extinção

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Abstract

For nearly forty years, at three institutions, our team conducted studies to advance the use of ARTs for propagating threatened and endangered mammalian species. The initial studies began in 1978 in the Department of Obstetrics and Gynecology at the University of Alabama in Birmingham, moving in the mid-1980's to The Center for Reproduction of Endangered Species (CREW) at the Cincinnati Zoo and lastly at the Audubon Center for Research in Endangered Species (ACRES) in New Orleans from 1996 to 2015. Our collaborative endeavors with more than two dozen zoological and academic institutions resulted in the births of ET offspring in two non-human primate species, four non-domestic bovid species and seven non-domestic felid species, six of which were inter-species transfers (Table 1). Origin of embryos that were successfully transferred ranged from those flushed from the uterus of mated females (baboon and bovids) to those generated by IVF, ICSI, and SCNT (gorilla and non-domestic felids). Additionally, embryos of five species underwent cryopreservation (baboon, common eland, African wildcat, caracal, black-footed cat) before successful transfer.

Keywords: assisted reproduction, primates, antelope, wild felids, endangered mammals

Introduction

From my early days on a farm, working with livestock, especially cattle and horses, was my primary interest. So, in college I majored in animal science as an undergraduate .Then, on the advice of a professor (and unofficial mentor) I went on to graduate school and majored in reproductive physiology (again, in the department of animal science). After post-doctoral study, I continued my domestic livestock pathway in the commercial cattle embryo transfer (ET) industry, which in the early 1970's could be appropriately defined as being in its "early stages of development".

In 1977, a serendipitous meeting with Lee Beck, a faculty member in the Department of Obstetrics and Gynecology at the University of Alabama in Birmingham (UAB) resulted in a major change in my career path from that of domestic livestock to the newly developing discipline of assisted reproduction technology in non-domestic, wild and endangered mammals. Although the primary funding of his research was for the development of controlled-release contraception systems using the baboon as the primate model, my directive was "do something with baboon embryos". As a first step, a commercially available uterine cell sampler was modified by adding micro-bore tubing to make it a continuous flow device that was used for transcervical embryo recovery in the baboon (Pope et al., 1980).

Non-domestic bovids

Meanwhile, from 1978 to 1985, as I worked mostly on developing methods for recovery, culture and cryopreservation of baboon embryos in Alabama I was also commuting to Ohio to do cow ET on a Holstein dairy farm near Cincinnati. At the same time, Betsy Dresser, at the Cincinnati Zoo, was establishing a program to develop assisted reproductive techniques (ART) for conservation of endangered species. Her request to use Holstein cows as recipients of common eland (*Taurotragus oryx*) embryos was the beginning of our 30 years of collaboration. For the first three years, the African antelope (common eland and bongo) ET trials at the zoo were synchronized with that of my Holstein cow ET schedule.

In Cincinnati, from 1983 to 1988, intraspecies ET was used successfully to produce calves in three species of antelope--common eland, bongo (*Tragelaphus eurycerus*) and scimitar-horned oryx (*Oryx dammah*). A signature accomplishment from this period was the successful transfer of bongo embryos recovered at the Los Angeles Zoo into eland and bongo recipients at the Cincinnati Zoo after a non-stop transcontinental flight that resulted in the births of two healthy bongo female calves—one from intra- and one from interspecies ET (Dresser et al., 1985). Neither recipient required obstetrical assistance during delivery, and both successfully raised their calves to maturity. Additionally, common eland calves were born after intraspecies transfer of fresh, frozen/thawed and demi-embryos (summarized by Pope and Loskutoff, 1998). The gestation length of common eland calves ranged from 271 to 278 days as compared to 285 and 290 days for bongo calves after inter- and intraspecific ET, respectively. Normally, gestation length of bongo is approximately one month longer than that of common eland.

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Also, in the late 1980's bovid interspecies ET resulted in the birth of a live gaur (*Bos taurus*) bull calf after non-surgical transfer of a late morula (1) and blastocysts (3) into four Holstein cows (Pope et al., 1988a). The live birth occurred as an unassisted delivery after a 10 mo gestation period, the usual length for gaur, but one month longer than is typical for Holstein cows.

The method of embryo recovery and transfer in bovids is predominantly determined by animal size and reproductive tract anatomy, particularly the cervix. The techniques used for transcervical uterine access in domestic cattle can be used with wild cattle and larger species of African antelope, such as eland and bongo; however, other approaches and/or equipment are generally necessary for small Bovidae. To avoid surgical exteriorization of the uterus for embryo recovery/transfer in species with which domestic cattle techniques are not possible, either laparoscopy or transcervical passage of small, stiff catheters with visual and digital guidance are used. Two species of antelope in which the latter technique was applied were the yellow-backed duiker (*Cephalophus sylvicultor,* Pope et al., 1988b) and scimitar-horned oryx (Pope et al., 1991). Fewer embryos were recovered from these two species, in part due to their small size and distinctive reproductive tract anatomy. Scimitar-horned oryx is one of several antelope species that has a duplex uterus in which the cervix is double, either completely or partially. In duiker, and some other antelope species, ovulation occurs from either ovary, but implantation is confined to the right horn (see Pope and Loskutoff, 1998). We nonsurgically transferred, using a ¼ cc Cassou AI catheter, a scimitar-horned oryx embryo at the morula stage to a synchronous 9 y old recipient who established pregnancy and delivered a live female calf after 247 days (Pope et al., 1991).

More than a decade after our early studies on ET in non-domestic bovids, we initiated a series of projects with the goal of advancing antelope ART into the 21st century. To achieve our primary objective of producing antelope (common eland and bongo) embryos in vitro required competence in a variety of disciplines, including, during the initial stages, behavioral conditioning of eland (Wirtu et al., 2005), transvaginal ultrasound-guided oocyte retrieval during mild sedation and, lastly, laboratory techniques for in vitro production of embryos (Wirtu *et al.,* 2009).

Another focus during this era involved developing a procedure for collection of antelope semen during standing sedation in a hydraulic restrain chute so that repeated samples could be obtained without general anesthesia (Wirtu et al., 2008). As a result, improved methods for cryopreservation of eland and bongo sperm were developed (Wirtu et al., 2006) that will be useful for future efforts at in vivo (AI) and in vitro (IVF) embryo production. The success of our efforts at collecting antelope gametes and deriving embryos in vitro was culminated when we did transvaginal endoscopic oviductal transfer of presumptive eland and bongo zygotes produced by intracytoplasmic sperm injection (ICSI) of oocytes recovered by transvaginal ultrasonographic-guided follicular aspiration (Wirtu et al., 2010). Unfortunately, pregnancy was not established in this initial and only trial.

Non-human primates

In Alabama, the first step toward the assigned goal of "doing something with baboon embryos" was devising a method to acquire them. Fortuitously, the materials for what turned out to the ideal device were already present in a lab that had been recently vacated by a biomedical engineer. As mentioned earlier, a commercially available uterine cell sampler modified by adding micro-bore tubing to allow for continuous flow was devised for transcervical embryo recovery (Pope et al., 1980). In the initial report, recovery rate (embryos and non-fertilized oocytes) increased from 35% for the first 40 flushes up to 58% for the second 40, to give an overall rate of 46% (37/80). In a subsequent multi-year study, recovery rate increased from 42% in year 1 to 52% in year 2 and 60% in year 3 (Pope et al.,1983a) suggesting that technician experience and knowledge of individual baboons were important influences on efficiency of the technique. Uterine embryos recovered from natural cycling females after mating ranged in development from two-cells to expanding blastocysts at one to six days after sex skin deturgescence. The recovery of 23 (8%) uterine embryos at the ≤ five cell stage suggests that some baboon embryos may arrive in the uterus at earlier stages of development than previously believed. Their developmental potential was not known, but a viable male offspring was born following nonsurgical ET of a four cell uterine embryo (Pope et al., 1983b). Embryos recovered at the six cell stage reached post implantation stages of development during in vitro culture (IVC), although at a reduced rate as compared to that of embryos recovered at later cleavage stages (Pope et al., 1982a). Also, live offspring were born after non-surgical transfer of cryopreserved embryos (Pope et al., 1984a; 1986).

Over a four-year period, 498 oocytes/embryos were recovered from 979 uterine flushes (51%) on 71 baboons (Pope et al., 1983a). Of 467 oocytes/embryos recovered from mated baboons, 290 (62%) were fertilized. *Papio anubis* females provided a higher percentage of embryos (75%) than did *Papio hamadryas* (48%) or *Papio cynocephalus* (44.3%) females following exposure to males during estrus. No embryos were recovered following 35 flushes on 11 (15%) baboons, although six of them were flushed only once or twice. Conversely, recovery rates for the ten most productive donors ranged from 66% to 93%, each of which yielded 12 to 33 oocytes/embryos. These ten most reliable donors provided 182 (37% of total) oocytes/embryos following 240 (76%) flushes, for an average of 18.2 oocytes/embryos per baboon.

With our individual embryo culture method and daily change to 'fresh' medium we examined secretion of some significant protein products of the peri-implantation conceptus in the absence of the maternal environment. Initially, we demonstrated that the capability for baboon chorionic gonadotrophin (bCG) production and secretion develops in the embryos during in vitro culture (Pope et al., 1982b). Additionally, during culture to post

implantation stages, embryos were shown to secrete pregnancy-specific β-1 glycoprotein (SP-1) into the culture medium (Pope et al., 1984b). Measurable quantities were usually present on Day 4 following attachment to the culture dish and continued for ≤ 14 days, with > 26 ug total SP-1 secretion from one embryo. Other pregnancy associated proteins that were studied included placental protein 5 (PP5) and pregnancy-associated plasma protein-A (PAPPA). Curiously, spent medium from post implantation baboon embryos demonstrated to secrete SP1 (Pope et al., 1984b) showed PP5, but not PAPP-A, immunoreactivity (by homologous monkey assays) (Sinosich et al., 1990).

Our visual observations during culture of embryos that appeared to have undergone differentiation of the inner cell mass into a bilaminar embryonic disc, with development of at least the primary yolk sac and amnion, were verified histologically (Pope et al., 1988c). The hypoblast, precursor to the primary yolk sac endoderm, was made up of an irregular mass of darkly staining, cuboidal. vesiculated and vacuolated cells reminiscent of previous descriptions of human embryos in vivo. The epiblast, from which the fetus will develop, consisted of a pseudostratified layer of columnar cells, with cells forming the lining of the amniotic cavity arising from its borders. The trophoblast in these embryos consisted of an outer layer of syncytiotrophoblast lined by paler staining cytotrophoblast. In some sections, the cytotrophoblast protruded through the syncytiotrophoblast in possibly early villous formation.

After a gap of 10 years since working with baboon embryos in Alabama, our lab in Cincinnati assembled a team from three local human infertility clinics to do a project on IVF/ET in a gorilla (*Gorilla gorilla gorilla*) (Pope et al., 1997). A 21 y old multiparous female with normal menstrual cycles was given hFSH (225 IU/d) and hCG (10,000 IU) to stimulate follicular development. At 35 h after hCG, follicles were aspirated under transvaginal ultrasound guidance. Metaphase II oocytes (n = 11) were placed in modified Human Tubal Fluid (mHTF, 100 uL) medium under oil at 37^{o}C in humidified 5% $C0_2$ in air. Frozen semen, collected by voluntary ejaculation, was thawed (5 sec air; 6 sec 70^{o}C H_20 bath), diluted slowly, centrifuged and resuspended in mHTF. Oocytes were inseminated at 6 h post recovery. After 21 h, eight oocytes were at the two cell stage, five of which were cryopreserved. Three were cultured to the six to eight cell stage in mHTF before transcervical uterine auto-transfer at 47 h post IVF. Ultrasound examination revealed a single fetus at 15 wk and unassisted delivery of a live 1.37 kg female infant occurred prematurely at 29 wk. This female "Timu", born in 1995 and the only greater ape produced from IVF/ET, is now 23 y of age and has lived most of her life at the Henry Doorly Zoo, Omaha, Nebraska. She has given birth to at least two live offspring.

Non-domestic cats

For most of the 30 years that our laboratory was involved in developing ARTs in domestic cats, we were applying the resultant technology and skills to several species of threatened or endangered cats. Our goal, besides using the domestic cat as a template, included taking advantage of their greater availability and genotypic and phenotypic similarities to several similarly sized wild relatives by doing interspecies embryo transfer. In the following section, I briefly summarize some of our studies applying ARTs developed using the domestic cat on several species of wild cats. The results in species in which live kittens were produced after intra- or interspecies ET will be described in more detail.

Indian desert cat (*Felis silvestris ornata*, IDC)

In the first project that incorporated interspecies ET in felids, we chose a close relative of the domestic cat, the Indian desert cat (Pope et al., 1993). A litter of two IDC kittens was born 68 days after IVF following uterine transfer of 55 IDC embryos into nine recipients. The kittens were born to a domestic cat recipient that had received both IDC (n = 4) and domestic cat (n = 10) Day 4 morulae.

African wildcat (*Felis silvestris lybica*, AWC)

Later, in New Orleans, working with the closest relative of the domestic cat--the African wildcat--a litter of three AWC kittens was born, but died in utero during parturition, after uterine transfer of 30 IVF embryos to three domestic cat recipients. After that, 22 Day 5 AWC IVF derived embryos cryopreserved in propylene glycol + sucrose were thawed and transferred to three domestic cat recipients, two of which established pregnancies and one live male kitten ('Jazz') was born on Day 70 (Pope et al., 2000). 'Jazz' was raised by the domestic cat recipient and died in 2016 at 17 years of age.

Our work with interspecies ET of AWC embryos into domestic recipients took a major step forward with studies on interspecies somatic cell nuclear transfer (iSCNT). Initially, the optimal combination of treatment conditions for maximizing the percentage of viable fibroblast cells at the G_0/G_1 phase were determined (Gómez et al., 2003). Thus, in subsequent SCNT experiments, the combination of serum starvation and pronase for cell cycle synchronization and cell separation, respectively, was used. Also, in vitro development of AWC cloned embryos reconstructed with cytoplasts from in vitro and in vivo matured oocytes was compared. Fusion rate was higher after using in vivo matured oocytes (97%) *vs*. in vitro matured oocytes (90%), but rate of blastocyst development did not differ—27% vs. 23%, respectively (Gómez et al., 2003). To determine the number of reconstructed AWC embryos

that should be transferred per recipient to consistently establish pregnancies (Gómez et al., 2004), SCNT embryos produced using 'Jazz' karyoplasts were transferred into domestic cat recipients within two groups: 1) recipients (n = 24) receiving ≤25 embryos (mean = 19.4), and 2) recipients (n = 26) receiving >30 embryos (mean = 41.7). Also, to determine the influence of length of in vitro culture on pregnancy rate, we compared laparoscopic oviductal transfer on Day 1 (n = 588) with uterine transfer of Day 5 (n = 467), Day 6 (n = 355) and Day 7 (n = 142) when embryos were at the one to two cell, morula and morula and blastocyst stage, respectively. Twelve of 26 (46.2%) recipients receiving >30 nuclear transfer (NT) embryos were pregnant when examined by ultrasonography at Day 21, while no recipients receiving ≤ 25 embryos were pregnant. Although no differences were observed in pregnancy rates after transfer of ≥ 30 embryos on Day 1 (6/12, 50.0%), Day 5 (4/9, 44.4%) or Day 6 (2/5, 40.0%) to synchronous recipients, more fetuses implanted after transfer of embryos on Day 1 (n = 17) *vs*. Day 5 (n = 4) or Day 6 (n = 3; $P < 0.05$). Six of seven (86%) pregnant cats with multiple fetuses were recipients of Day 1 SCNT embryos (Gómez et al, 2004)

In addition to demonstrating that embryos generated by SCNT of AWC karyoplasts with domestic cat cytoplasts could develop to the blastocyst stage and into live kittens after interspecies ET into domestic cat recipients, Gómez et al. (2008) then reported that sand cat (SC, *Felis margarita*) kittens likewise could be generated by ET of iSCNT embryos into domestic cat (DSH) recipients (see Table 1). Subsequently, in 2009, Gómez et al., summarized her studies on in *in vitro* and *in vivo* developmental competence of SCNT embryos from four small felid species [AWC, SC, black-footed cat (BFC, *Felis nigripes*) and rusty-spotted cat (RSC, *Prionailurus rubiginosus*)] that were reconstructed with heterospecific domestic cat cytoplasts and transferred into domestic cat recipients. Cleavage frequency of reconstructed embryos was not affected by differences in species or genera between donor cells and recipient oocytes, but blastocyst development was lower for interspecies SCNT embryos reconstructed with BFC and SC fibroblast cells than in the other species. Unexpectedly, blastocyst development of RSC–DSH embryos reconstructed by intergeneric SCNT was similar to that of interspecies AWC– DSH SCNT embryos. Even though fewer BFC–DSH and SC–DSH cloned embryos developed to the blastocyst stage, pregnancy rates and implantation rates after transfer into DSH recipients were similar to those seen after transfer of AWC–DSH SCNT embryos. Nonetheless, all implanted BFC– DSH cloned embryos were reabsorbed by mid-gestation. Transfer of intergeneric RSC–DSH cloned embryos into DSH recipients failed to produce any pregnancies.

Table 1. Offspring born after embryo transfer in non-human primates, non-domestic bovids and non-domestic felids from 1983 to 2013 at the University of Alabama in Birmingham (UAB), Cincinnati Zoo and Audubon Nature Institute, New Orleans.

Donor	Recipient	Embryo source	Method of Transfer	Offspring		Year	Location
				Frozen	Fresh		
Baboon	Baboon	In vivo, uterine	transcervical	1	3	1983-4	University of Alabama, B'ham
Common eland	Common eland	In vivo, uterine	transcervical	2	2	1983-5	Cincinnati
Bongo	Common eland	In vivo, uterine	transcervical	1	--	1984	Cincinnati (Los Angeles)
Bongo	Bongo	In vivo, uterine	transcervical	1	--	1984	Cincinnati (Los Angeles)
Scimitar-horned Oryx	Scimitar-horned oryx	In vivo, uterine	transcervical	1	--	1989	Cincinnati
Gaur	Domestic cow	In vivo, uterine	transcervical	1	--	1987	Cincinnati
Indian desert cat	Domestic cat	IVF	Laparotomy uterus	2	--	1989	Cincinnati
Gorilla	Gorilla	IVF Frozen sperm	transcervical	1	--	1995	Cincinnati (Omaha)
African wildcat	Domestic cat	IVF	Laparotomy uterus	2	1	1999	New Orleans
Caracal	Caracal	IVF, Frozen sperm	Laparotomy uterus	2	3	2000-1	New Orleans
Fishing cat	Fishing cat	IVF	Laparotomy uterus	1	--	2006	New Orleans
Serval	Serval	IVF	Laparotomy uterus	1	--	2006	New Orleans
Black-footed cat	Black-footed cat	IVF Frozen sperm	Laparoscopy oviduct	--	2	2011	New Orleans (Omaha)
Black-footed cat	Domestic cat	IVF Frozen sperm	Laparoscopy oviduct	--	1	2012	New Orleans (Omaha)
African wildcat	Domestic cat	iSCNT	Laparoscopy oviduct	8 (+9 died <30 days)	--	2003	New Orleans
Sand cat	Domestic cat	iSCNT	Laparoscopy oviduct	1 (+13 died ~ birth)	--	2008	New Orleans (Birmingham)
Caracal	Caracal	SCNT	Laparoscopy oviduct	1	--	2010	New Orleans

From 1999 to 2012, non-domestic kittens following ET of IVF derived embryos were produced in four other species-caracal, fishing cat (Pope et al., 2006a), serval (*Leptailurus serval*; Pope et al., 2006b) and black-footed cat (*Felis nigripes,* Pope et al., 2012). Also, during this period, SCNT kittens were born after intra- and inter species ET in two other species-caracal (Gómez and Pope, 2015) and sand cat (*Felis margarita;* Gómez et al., 2008*), respectively.* Some of those studies are briefly described in the following section.

Fishing cats (*Prionailurus viverrinus*)

From five females (three to six oocyte retrievals/female), a total of 574 preovulatory oocytes were recovered during 21 gonadotropin treatments/oocyte retrievals (mean = 27.3). An average of 30 and 25 oocytes were recovered/retrieval at the first two retrievals (n = 10 retrievals) and the third through sixth retrievals (n = 11) of each female, respectively. Fishing cats showed both a high degree of follicular receptiveness to exogenous gonadotropins and a more than usual similarity among females. Eleven ETs were done on Day 5 (n = 9) or Day 6 (n = 2) after oocyte retrieval. Day 5 or Day 6 IVF-derived embryos (n = 127, mean = 12.1) were auto-transferred to the uteri of four females (one to four ETs/female). One pregnancy (9%) was obtained after transfer of 10 Day 6 embryos derived by IVF with sperm stored for 24 h at 4°C. A live female kitten was born without assistance on Day 63 of gestation (Pope et al., 2006a)

Caracal (*caracal caracal*)

From six to eight gonadotropin treatments/oocyte retrievals/female were done in three females, one of which produced below average numbers of preovulatory oocytes at two retrievals and only immature oocytes at five retrievals. Two females produced preovulatory oocytes at every retrieval except one, from which an average of 33.1 and 24.7 oocytes were recovered during retrievals one through three and four through six, respectively. Thus, both fishing cats and caracals show repeated follicular receptivity for to up to six to eight gonadotropin treatments. The slight decline in the average number of oocytes recovered from both species after the third treatment/retrieval is probably due to a combination of factors, including attenuation of ovarian receptiveness and increasing age of females during the 5 years of the study. After the birth of a litter of two live female caracals by transferring IVF derived embryos into six recipients, we began cryopreserving embryos on Day 5 of IVC for later transfer. Then, cryopreserved embryos (n = 70) were thawed and transferred to six Day 5 recipients or placed in culture until Day 6 when they were transferred to one Day 5 (n = 10) or two Day 6 (n = 29) recipients. Three pregnancies (33%) were established and a total of three kittens were born. Two pregnancies were obtained from synchronous Day 5 transfers and one from asynchronous transfer of Day 6 embryos into a Day 5 recipient. Embryos transferred in the four caracal ET pregnancies were produced using IVF with frozen/thawed sperm (Pope et al., 2006a).

Black-footed cat (Felis nigripes)

A total of 165 oocytes was recovered from six donors during 14 oocyte retrievals (mean = 11.8). An average of 12.2 oocytes (2 to 25) was recovered at the first oocyte retrieval as compared to 12.7 oocytes (9 to 22) from five females at the second. Cleavage frequency of oocytes after IVF with cooled or cryopreserved sperm was 38/54 (70%) and 50/106 (47%), respectively ($P < 0.01$). Four intraspecies laparoscopic oviductal ET procedures were done on Day 1-two recipients received fresh Day 2 embryos (n = 5, 8) and two recipients received embryos that were cryopreserved on Day 1 (n = 6) or Day 2 (n = 8). At ultrasonographic examination on Day 50, the 2 y old BFC recipient of Day 1 cryopreserved embryos was determined to be pregnant and delivered two live male kittens, without assistance, on Day 69. One year later, five cryopreserved BFC embryos, cohorts to those that resulted in the two kittens born after intraspecies ET, were transferred by laparoscopy into the oviduct of a 7 mo. old domestic cat recipient on Day 1 after oocyte retrieval. Ultrasonographic examination on Day 29 revealed one fetus and a live female BFC kitten was delivered without assistance on Day 66. The kitten weighed 65 g and 159 g at birth and at 15 days of age, respectively. In comparison, the two male BFC kittens born after intraspecies ET weighed 156 and 198 g when first examined at 15 days of age (Pope et al., 2012).

In New Orleans we worked with a total of nine species of wild cats, including the smallest (sand cat, black-footed cat, African wildcat), mid-sized (serval, caracal, clouded leopard, fishing cat) and the largest (lion and tiger). As described above, from the nine species, we produced live ET offspring in six (African wildcat, caracal, serval, fishing cat, black-footed cat, sand cat), including kittens from ET of cryopreserved embryos in three (African wildcat, caracal, black-footed cat) and kittens from ET of SNCT embryos in three (African wildcat, sand cat, caracal). Additionally, we did ET of IVF (or ICSI) derived embryos in three others—clouded leopard, lion (ICSI) and tiger—that did not result in pregnancies and are described below.

Clouded leopards ((Neofelis nebulosa)

Eight females 4 to 10 y old at our first treatment were administered 15 or 20 IU of porcine FSH over four days. One, two or three laparoscopic oocyte retrievals were done 24 h after porcine LH treatment (15 or 20 IU) on

three, three and two females, respectively (total = 15), 10 of which (67%) were done on females that were eight to 12 y of age. A total of 176 preovulatory oocytes (mean =14.7) were recovered from 12 oocyte retrievals on six females. Two females, a 5 y old and an 11 y old, did not respond to gonadotropin treatment. Of five females with ≥ two oocyte retrievals an average of 16.2 and 14.6 oocytes were recovered at the first and second oocyte retrievals, respectively. Semen from three males was used after cryopreservation or after storage at 4°C for 24 h (cooled). Cleavage frequency was 43% overall, 55% (64/117) after IVF and 20% after ICSI with frozen (10/43) or cooled (2/16) sperm. After IVF with frozen sperm, cleavage rate was 63% (48/76) *vs*. 39% (16/41) with cooled sperm. Twenty-four Day 5 cryopreserved embryos were thawed and transferred, by laparotomy, to the uteri of three Day 5 gonadotropin-treated recipients (seven to nine embryos/female). Similarly, 28 Day 5 fresh embryos were auto-transferred to three Day 5 recipients (five to 15 embryos per female). Most embryos transferred were morulae (83%) produced by IVF using frozen sperm (71%). No recipients established pregnancies, undoubtedly, a multi-faceted failure. Age may have been the primary factor as most recipients were 8 to 11 y old. Nonetheless, the results showed that clouded leopard females were responsive to exogenous gonadotropins and that embryos could be consistently produced in vitro, both by IVF and ICSI, using both cryopreserved and cooled sperm (Pope et al., 2010).

Lion (Panthera leo)

We did a preliminary trial in applying ICSI to in vivo matured lion oocytes. Laparoscopic oocyte retrievals in three gonadotropin-treated lionesses produced a total of 38 oocytes, most of which had extruded the first polar body and were injected with cryopreserved epididymal sperm obtained by percutaneous epididymal sperm aspiration (PESA) from a 12 y old vasectomized male. More than 60% of sperm-injected oocytes cleaved (n = 17) and developed to the morula stage and were cryopreserved in propylene glycol using a slow controlled cooling rate. Later, the ICSI derived embryos were thawed and transferred to a 14 y old lioness who had exhibited a natural estrus, but she did not establish pregnancy (Damiani et al., 2004).

Tiger (Panthera tigris)

To do a collaborative project with the Baton Rouge Zoo in 1998 it was necessary to mobilize our equipment for laparoscopic oocyte retrieval, oocyte handling and incubation during transport. Five females (four Bengal, one Siberian) were treated on seven occasions with porcine FSH for four days. Porcine LH was administered on Day 5 and laparoscopic oocyte retrievals were done 25 h later. Recovered oocytes were transported in a portable incubator to the lab in New Orleans (150 km) where oocytes with expanded cumulus cell masses were co-incubated with frozen/thawed sperm (1 to 2 x 10^5/ml) for 12 to 15 h. At 40 h post-insemination, cleavage frequency was determined and embryos at the two to eight cell stage were frozen in 1.4 M propanediol and 0.125 M sucrose. A total of 325 mature oocytes were recovered during seven laparoscopic procedures (mean = 46.4) and from these, 171 embryos (mean = 24.4) were produced for a mean cleavage frequency of 53%. Most likely, cleavage after IVF of oocytes from four females was compromised by poor quality of semen samples from the Bengal male. The results of this initial report demonstrated that tiger embryos could be produced reliably in vitro after ovarian follicular stimulation by daily treatment with exogenous gonadotropins, off-site laparoscopic follicular aspiration and transport of oocytes to the ART laboratory for IVF with frozen sperm (Pope et al., 1999)

Summary

My career of developing assisted reproductive technologies in non-domestic, wild and endangered mammalian species began in 1978, coincident with the birth of Louise Brown, the first human born after transfer of an IVF derived embryo. At that time, the only other IVF derived births had been in a few species of lab animals and births after ET of cryopreserved embryos had been achieved in only three species—mouse and rabbit and domestic cow. The decade of the 1980's saw a tremendous expansion of interest in applying ARTs in a wide range of areas, ranging from research (basic reproductive mechanisms), to commercial (ET in domestic livestock, especially cattle), to infertility (humans). The rapid advancements and success achieved in these areas combined with an increasing awareness of the importance of conserving our wild fauna and flora (including Endangered Species Act, 1973, U.S. Fish and Wildlife Service) gave rise to the establishment of ART laboratories at a few larger zoological institutions. Among those were the Center for Reproduction of Endangered Species (CREW) at the Cincinnati Zoo in the mid-1980's and was followed by the Audubon Center for Research in Endangered Species (ACRES) in New Orleans during the following decade.

For nearly forty years, our team conducted studies to advance the use of ARTs for propagating threatened and endangered mammalian species. Our collaborative endeavors with more than two dozen zoological and academic institutions resulted in the births of ET offspring in two non-human primate species, four non-domestic bovid species and seven non-domestic felid species, six of which were inter-species transfers (Table 1). Origin of embryos that were successfully transferred ranged from those flushed from the uterus of mated females (baboon and bovids) to those generated by IVF, ICSI, and SCNT (gorilla and non-domestic felids). Additionally, embryos of five species underwent cryopreservation (baboon, common eland, African wildcat, caracal, black-footed cat) before

successful transfer.

This abbreviated overview of our ART studies during the past four decades focused primarily on the embryo transfer aspects. What is not described are the ancillary lab techniques that were required for producing embryos in vitro, including optimizing the culture environment for in vitro oocyte maturation, in vitro fertilization (IVF and ICSI) and in vitro embryo culture and cryopreservation of oocytes and embryos (Pope, 2000; Pope *et al.,* 2006c and companion proceedings paper on 'ART in domestic cats'). Equally important were the studies done on sperm collection techniques, handling and storage—both short-term cool and long-term cryogenic. It could be asserted that the most important ART involving ET is recovery/retrieval of oocytes/embryos from the donor female. As an animal welfare consideration, the methods must present minimal short- and long-term health risks. Moreover, because of genetic significance and nominal numbers of animals, the capability to do multiple procedures on select individuals while conserving ovarian response to exogenous gonadotropins is a key factor (Pope *et al.,* 2006a; Pope, 2014).

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